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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

JAN 30 1974

Application for Research Grant
(Use extra pages as needed)

Date: 1/10/74

1. Principal Investigator (give title and degrees):

Dov Michaeli, Ph.D. Assistant Professor of Biochemistry & Surgery
Co-investigator: H. Hugh Fudenberg, M.D., Professor of Medicine, Professor of Bacteriology and Immunology

2. Institution & address:

University of California San Francisco
School of Medicine, Room 839 HSE
San Francisco, California 94143

3. Department(s) where research will be done or collaboration provided:

Departments of Biochemistry and Surgery and the Department of Medicine

4. Short title of study:

Effects of Cigarette Smoke on Pulmonary Fibroblasts and Collagen and its Relation to Emphysema

5. Proposed starting date: 3/1/74

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

1. Study the immunogenicity of cigarette smoke components complexed with lung collagen by the radioimmunoassay (RIA) technique.

2. To study the effects of components of the gaseous phase of cigarette smoke on the division rate of lung fibroblasts, and on the synthesis and secretion of collagen and mucopolysaccharides.

3. Identify the cellular site(s) of action of cigarette smoke components

4. Study the chemical interactions between cigarette smoke components and matrix macromolecules, and the possible effect of such interactions on the biosynthetic activity of lung fibroblasts.

5. Study the immunopathology of emphysematous lungs using immuno-electronmicroscopic techniques.

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8. Brief statement of working hypothesis:

We have found (1) that approximately 70% of emphysema patients had antibody titers to denatured human collagen. In our search for a possible mechanism for the induction of such antibodies it became necessary to examine the effects of cigarette smoke under controlled conditions. We therefore developed a tissue culture system of human lung fibroblasts (WI-38) exposed to various concentrations of smoke components. We observed that at low concentrations (0.108 μg/ml) formaldehyde stimulated the synthesis and secretion of collagen and hyaluronic acid. At higher concentrations, formaldehyde caused a marked decrease in synthesis of connective tissue macromolecules. The mechanism for these effects may be due to a direct effect on the fibroblast and/or on the matrix macromolecules, which in turn causes an aberration of the signals received by the cells from the extracellular environment. A major hurdle in studying the effects of cigarette smoke on fibroblast in culture under controlled environmental conditions was the inadequacy of currently available assays for measuring the low concentrations of newly synthesized collagen. We have recently solved this problem by developing an extremely sensitive radioimmunoassay assay technique (2). This makes the stated aims of this proposal extremely feasible and realistic.

9. Details of experimental design and procedures (append extra pages as necessary)

1. Immunogenicity of collagen following its interaction with smoke components

The breakdown of tolerance to self-proteins through their chemical modification is a well-established phenomenon. Weigle (6,7) injected modified rabbit thyroglobulin into rabbits and created an autoimmune thyroiditis resembling Hashimoto's disease in humans. The possibility that interaction of cigarette smoke components with lung collagen may induce an immunologic reaction against this organ is very attractive. We have described the presence of antibodies to denatured collagen in 70% of emphysema patients, by the hemagglutination technique (1). However, these antibody titers may actually reflect a cross reaction of antibodies whose homologous antigen is lung collagen modified by smoke components. In order to evaluate this possibility, a much more sensitive and quantitative assay was required. Hence, a major research effort was invested in developing a RIA for collagen. We recently succeeded in perfecting such an assay and are capable of measuring 10^{-9}g (nanogram) quantities of collagen. This gives us the capability to measure:

1. the amount of circulating collagen in sera of emphysema patients, compared to normal sera and sera of other lung diseases, and

2. measure the amount of antibodies in sera of emphysema patients directed against lung collagen and against lung collagen modified by cigarette smoke components.

If indeed such a reaction can be demonstrated, isolation and identification of the newly created antigenic determinants will be of prime interest. The best approach would be to cleave the collagen with CNBr (8) and test the peptides for RIA. The significance of the immunological studies in general and the identification of the antigenic determinants in particular, will be discussed below.

2. Effects of cigarette smoke on lung fibroblasts

This study is already well underway. The following is a representative example of results obtained thus far.

9. Experimental design and procedures (continued)

Cigarette smoke from one non-filter cigarette was passed through 50 ml. of Dulbecco media. The media was then diluted to dilutions ranging from 1:5 to 1:10,000. Assuming 15 puffs/cigarette, then 5 ml (the volume used in tissue culture dishes) of undiluted medium represents 1.5 puffs ($\frac{15 \times 5}{50}$), and diluted media represents a proportionate fraction thereof.

When lung fibroblasts (WI-38) were added to smoke-treated media many of the cells failed to attach to the dish. After a 2 day growth period the cells were pulse labeled with H^3-thymidine and the amount of radioactivity uptake was determined. The results (Table I) indicate that extremely minute quantities of cigarette smoke can inhibit completely the mitotic activity of lung fibroblasts.

When treated media was added to established cultures, the cells were more resistant to cigarette smoke but were still substantially affected by the media.

Table I

Effect of media treated with cigarette smoke on division activity of human lung fibroblasts (WI-38)

Treatment	Dilution of treated media	H^3-thymidine uptake (cpm)	% inhibition
I Control (un-treated media	-	5801	0
II Fibroblasts added to smoke treated media	1 (undiluted)	72	98.8
	1:5	331	94.3
	1:10	1666	71.3
	1:50	3335	42.6
	1:100	5738	1.0

Our next step was to incorporate components of the gaseous phase of cigarette smoke in tissue culture media and add it to established fibroblast culture. The parameters measured were multiplication rate (measured by incorporation of H^3-thymidine), synthesis, and secretion of collagen (measured by RIA), and synthesis and secretion of mucopolysaccharides (measured by incorporation of $S^{35}O_4^=$ or C^{14} sodium acetate). In general, formaldehyde exhibited the most profound effects with acetaldehyde next and propionaldehyde exhibiting only slight effects. No effect was exhibited by acetone, butanone and nicotine, at the concentrations tested. The concentrations added to the tissue culture medium were based on Kensler and Battista's analysis (2) of

the composition of the gaseous phase of cigarette smoke. The results (Table 2) indicate several facts.

First, the injurious effects of aldehydes are in inverse relationship to the length of the alkyl group. Thus, although formaldehyde occurs in cigarette smoke in much lower concentrations than acetaldehyde, the former is by far the most injurious both relatively (per mole or μg) and absolutely (per cigarette). Secondly, close examination of the data reveals an interesting relationship between dose of aldehyde and its effect of fibroblasts. When fibroblasts were added to formaldehyde-or acetaldehyde-treated media, many cells either failed to attach or were more sensitive to the toxic effects of aldehydes after their attachment; this is reflected in a progressive decrease in the protein content/culture dish. The surviving cells in the formaldehyde-treated media also synthesized less collagen. A situation that much more closely resembles conditions prevailing in vivo occurs in treatment II, where aldehyde-treated media is added to established and confluent fibroblast cultures. At low levels of formaldehyde (0.108 μg/ml, equivalent to a concentration present in 0.03 puffs of cigarette smoke) there was an increase in total protein (122% of control) and a marked increase in collagen synthesis (255% of control), while no change occurred in the synthesis of mucopolysaccharides. At a higher concentration (1.08 μg/ml) the toxic effects of formaldehyde supervene and there is a general decrease not only in total protein (reflecting the number of surviving cells) but also in the synthetic rate of the surviving cells. These results provide only a glimpse of the potential that our tissue culture system provides for elucidation of biochemical mechanisms involved in the formaldehyde effects on cells, and for quantifying the dose-response relationship.

A third point of interest in our data is the effect of acetaldehyde. Although it is present at much higher concentrations in cigarette smoke, its effects on lung fibroblasts are much milder. Because of that, higher concentrations of acetaldehyde (43 μg/ml), added to confluent cultures, activate whatever mechanisms are responsible for the shift toward increased synthesis of collagen ($>$ 1000 ng) to an even higher degree than formaldehyde did. The significance of this observation in understanding the pathophysiology of certain fibrotic reactions in the lung are discussed below.

An interesting observation which is outside the realm of this application, is the progressive increase in thymidine uptake (i.e. proliferation rate) of fibroblasts when added to media containing acetaldehyde (Table I, treatment I). Is there a possibility that continuous maintenance of cells in contact with acetaldehyde would result in the eventual "escape" of some cells from the internal controls on proliferation rate, leading to an uncontrolled, neoplastic growth? Such a study would require long term experiments which are not contemplated in this proposal.

These results are probably merely two of the multitude of parameters that distinguish between the effects of moderate and high concentrations of formaldehyde and other aldehydes in cigarette smoke. Two of the most important parameters that we plan to measure are: 1) Effect of aldehydes on cell

TABLE 2

Treatment	Agent	Conc. µg/ml	Puffs equiv.	Protein Total	Protein % of Control	Thymidine Uptake (cpm) Total	Thymidine Uptake (cpm) µg Protein	Thymidine Uptake (cpm) % of Control	Collagen Synthesis (ng) Total	Collagen Synthesis (ng) µg Protein	Collagen Synthesis (ng) % of Control	MPS Synthesis (cpm) Total	MPS Synthesis (cpm) µg Protein	MPS Synthesis (cpm) % of Control
I. Control (fibroblasts added to normal media)	-			612		3250	5310		258	421				
Fibroblasts added to treated media	Formaldehyde	0.011	0.003	504	82.3	3149	6248	117.6	197	390	92.6			
		0.108	0.03	568	92.8	3212	5654	106.4	110	193	45.8			
		1.08	0.3	60	9.8	181	3023	56.9	N.D*					
	Acetaldehyde	0.432	0.003	588	96.0	3565	6062	114.1	147	250	59.3			
		4.32	0.03	584	95.4	4012	6869	129.3	148	253	60.0			
		43.2	0.3	340	55.5	3017	8873	167.0	127	373	88.5			
II. Control (normal media added to confluent fibroblast culture)	-			752		990	1316		320	425		8,177	10,873	
Treated media added to confluent fibroblast culture)	Formaldehyde	0.108	0.03	920	1223	1120	1217	92.4	1000	1087	255	10,062	10,966	101
		1.08	0.3	444	59.0	628	1414	107.4	138	310	73	3,773	8,497	78
	Acetaldehyde	4.32	0.03	944	125.5	952	1008	76.5	250	264		9,993	10,585	197
		43.2	0.3	N.D.*		735			>.1000			7,624		

* Not Done

survival; (we already have qualitative observations that increasing concentrations of aldehydes cause increased cell death). 2) Effect of aldehydes on the length of fibroblast cell cycle. For this purpose it is imperative to work with synchronized cultures, and recently we have started such a culture using methods we used previously for synchronizing lymphocytes in *in vitro* culture (21).

3. Cellular sites of action of smoke components

These studies are designed to identify the mode(s) of action of aldehydes and other smoke components on the cellular level.

Labelled aldehydes will be added to the culture media and incubated with the cells for various time intervals. The labelled media will then be removed and the cells incubated with the corresponding unlabelled aldehyde to chase the unbound labelled agent. Cells will then be fractionated (22) and the various fractions counted.

We have already begun these experiments and preliminary results indicate that the cell membrane is the primary site of accumulation of formaldehyde; after 30 minutes of incubation 14% of the incorporated radioactive material was found in the membrane fraction, and after 24 hours incubation-40% was found in that fraction. These results are preliminary and a thorough study of all cell organelles and fractions, obtained by differential centrifugation in a sucrose gradient, is planned.

4. Interaction between cigarette smoke components and extracellular matrix

It is well known that the biosynthetic activity of mesenchymal cells is controlled to a large extent by signals from the extracellular environment (for review see 3). One of us (Z.N.) has demonstrated (4) that the concentration of chondromucoprotein in the matrix controls the rate of further synthesis by chondrocytes. We have recently been engaged in investigating the extracellular signals controlling the rate of synthesis of collagen and hyaluronic acid. It is quite likely the chemical interaction between the various aldehydes in cigarette smoke and collagen might cause a distortion in the signals received by the cells. For instance, we have found that the rate of collagen synthesis is inversely related to the extent of collagen cross-linking in the immediate environment of the fibroblast. Since formaldehyde and acetaldehyde are known cross-linking agents, it is conceivable that their effect would be to reduce collagen synthesis by lung fibroblasts.

We are proposing to react collagen with labelled formaldehyde, so that the extent of interaction could be quantitated. Also, the degree of cross-linking will be measured directly. This will be done by denaturation of the treated collagen at 50°C for 10 minutes and chromatography on a CM-cellulose column, according to the method of Piez et al (5). The ratio of β components to α monomer chains will serve as an accurate index for the degree of cross-linking.

Lung fibroblasts will be incubated in media containing either H^3-proline,

C^{14} glucosamine, or $S^{35}O_4^{=}$, and in the presence of collagen cross-linked to various degrees. The rate of collagen synthesis will then be measured by the rate of H^3HyPro secretion into the media and/or by a RIA of collagen developed in our laboratory; the rate of sulfated mucopolysaccharide synthesis will be measured by the rate of C^{14} or S^{35} Hyaluronic acid secretion. Thus, this experiment, for the first time, will enable us to correlate the extent of collagen cross-linking with the rate of fibroblast synthetic activity, and more important for this proposal--will provide a basis for quantitation of the exposure to cigarette smoke and the effects of such exposures on the synthesis of new lung tissue.

5. Immunoelectronmicroscopy

Electron microscopic studies during the past three years (summarized in the Progress Report) indicate that profound ultrastructural changes occur in lungs of patients with emphysema (12) and in rodents with experimental emphysema (13) induced by antigens which inhibit collagen cross-linking. Common to and prominent among these changes in both man and rodent is loss of organization of collagen fibrils as shown by freeze-etch and freeze fracture electron microscopy.

To ascertain whether these changes are accompanied by and presumably due in part to immunologic events, antibody localization studies at the ultrastructural level are necessary to show that antibody to antigen are present at the site of the lesion. Such studies will also prove helpful in confirming any differences in antigen density found by the immunofluorescent technique (*vide infra*).

Lung tissues from smokers and non-smokers, emphysematous and "control" subjects will be obtained from pulmonary resection for other causes, and from autopsy. Tissue from animals with emphysema induced by β aminopropionitrile (13) will be obtained. With anti-collagen-anti-ferritin hybrid antibodies the relative numbers of native collagen/altered collagen sites per cell can be obtained, and correlation attempted with severity of disease (as measured by various pulmonary function tests), age, smoking history, and other parameters.

Comparable studies in β aminopropionitrile-induced emphysema in rodents, if producing identical results, will almost unequivocally demonstrate this to be a valid animal model for pulmonary emphysema.

Antibodies to human IgG, to collagen and to ferritin have already been prepared. Antibody to aldehyde-collagen complex is in the process of being prepared.

Hybrid antibody (one end directed to ferritin, the other end to IgG or to collagen) will be prepared by the method of Fudenberg, Drews, and Nisonoff (14).

Addition of ferritin-collagen hybrid antibody to cells will result in the anticollagen site of the divalent antibody binding to the tissue, and the other site remaining exposed at 180 angstrom from the tissue antigen site; this is the best antibody method available for localization and enumeration of antigen sites per cell and has been used, for example, by Hammerling, et al (J. Exp. Med., 128, 1461, 1968), to enumerate the number of and to localize the sites of, the antigens in mouse thymocytes. It is vastly superior to use of native antibody labelled with ferritin or horse-radish peroxidase. Use of hybrid antibody to ferritin and to IgG will permit enumeration of the number of IgG molecules bound/cell. If these are all antibodies to native or all to aldehyde-collagen complexes, the number of molecules detected with the anti-IgG--anti-ferritin hybrid should be identical. If a portion of the emphysematous lung sites react with the anti-native collagen, and another portion with anti-aldehyde-collagen complex, data on relative ratios in various patients will be correlated with severity of disease (assessed by pulmonary function tests), duration of disease, smoking history, etc.

Significance

Achievement of the goals that we have set to ourselves will result in a better understanding of the relationship between cigarette smoke and emphysema. It may also offer a new approach to a sorely lacking means of early detection of the disease. Specifically, the four broad lines of investigation proposed herein have the following goals:

1. The study on the effects of various concentrations of aldehydes on cell division, life span, and synthetic activity may provide insights into the etiology and pathophysiology of emphysema. The slow disappearance of connective tissue components from the affected lung may be the result of normal turnover without the proper re-placement. Such a process would result from killing of fibroblast and, at higher concentrations of formaldehyde, reduction of the synthetic activity of the surviving cells. Moreover, the observation that moderate levels of formaldehyde can cause an increase in the synthesis of collagen may explain the epidemiologic observation that among workers exposed to cotton dust (9) or to asbestos fibers (10), pulmonary fibrosis is more frequent and more extensive when cigarette smoking is added to the dust exposure.

2. Identification of the cell fraction(s) to which aldehydes bind will lay the groundwork for understanding the mode of action by which they exert their injurious effects.

3. The study of the interaction of smoke components with lung matrix molecules and study of the immunogenicity of these complexes may provide a basis for understanding the immunogenicity of lung collagen in emphysema patients, as illustrated by the fact that 70% of these patients have antibodies against collagen. This may also serve as a new approach to development of a test for early detection of emphysema. A key requirement for the latter two goals

is the availability of a sensitive and quantitative assay for collagen. This we have already accomplished by the development of radioimmunoassay of collagen.

Fluorescent Antibody Studies

In contrast to the main body of the application, part of which has already been carried out, this part is more speculative and we are unsure about its outcome. We therefore propose it as a separate part, and are asking for only minimal funding to try it. If the idea proves successful, an expanded proposal along this line of investigation will be submitted.

Background

As statedpreviously in this application, we have found antibodies to collagen in 70% of patients with emphysema. If 1-5% of patients with emphysema develop the disease as a result of homozygosity for a gene producing protein inhibitor (alpha-antitrypsin, anti-collagen, and anti-elastin) deficiency, this leaves another 25-29% unaccounted for, suggesting that in the subject with normal Pi levels at least two separate etiologic mechanisms exist.

Objective

We propose to ascertain whether the two types are due to (a) differences in immunologic host defense, and/or (b) to differences in antigen content per cell in the pulmonary parenchymal cells in these two types.

We also propose to investigate lung tissues of smokers and non-smokers (obtained during pulmonary resection for other causes, with autopsy) to discern whether antigen content/cell in smokers without emphysema differs from the normal non-smokers and from smokers with emphysema.

Further, although anti-collagen antibodies are presumably the result rather than the cause of the emphysema, it seems possible that once present, they may contribute to further lung damage in a genetically predisposed host, thus creating a vicious cycle. This also will be investigated.

Materials and Methods

The major difficulties with fluorescent microscopy (as ordinarily used) as applied to studies of the lung are:

1) Fixation of lung tissue for adequate spatial localization.

2) Inability to quantitate the intensity of fluorescence.

During the past two years, we have:

1) Developed techniques for fixation of lung tissue for immunofluorescence and immunoelectronmicroscopy without loss of spatial relationship.

2) Developed a technique, utilizing a Leitz Orthoplan fluorescent microscope with incident lighting and an MDVI Spectrophotometer

with automatic read-out. This technique allows us to localize and quantitate (0-100% rather than 1+ to 4+) antibodies directed against single cells in tissues, (by automatic subtraction of background fluorescence) and to estimate differences in antigen density between cells from homologous tissues (16).

Antibodies to native collagen and denatured collagen have already been proposed, and those to collagen altered by various components of cigarette smoke are being proposed. These have been and will be labelled with fluorescent isothiocyanate and rhodamine by the methods used previously (17). All antisera will be characterized as to the peptide in collagen which bear the corresponding homologous antigenic determinant.

Pulmonary cells from non-smokers and smokers "normal", and from smokers and non-smokers emphysematous lung, will be compared with various antibodies by indirect immunofluorescence. Direct fluorescence will also be done to ascertain anti-collagen antibody bound in vivo.

Significance

1) Increased antigen density as revealed by the same antisera presumably renders a cell more liable to destruction by antibody than one with decreased numbers of antigen sites. Cigarette smoke aldehydes may pathologically act not only to cause destruction of pulmonary cells, but perhaps also to revive "buried" determinants by disabling a portion of the cell membrane (similar to the action of various proteolytic enzymes in exposing additional antigens on the red cell membrane (e.g. 18, 19). Similar studies will also be performed on cell lines of normal lungs in an in vitro culture before and after exposure to various cigarette aldehydes.

If such is indeed the case, antigenic density/cell in emphysematous lung as compared to that of non-emphysematous lung should be greater. Such comparison will be especially useful in lung tissue obtained from emphysematous and non-emphysematous subjects with comparable smoking histories matched for age, sex and ethnic origin.

If "normal" smokers, with few exceptions, show no differences as compared to normal non-smokers, perhaps a prognostic tool can be devised to predict those liable to develop emphysema (on the basis of similar studies on cells obtained from bronchial aspirators for cytologic studies), and thus council only those liable, presumably for genetic reasons, to develop the disease to avoid cigarette and other possibly deleterious environmental factors.

2) Alternatively, various aldehydes found in tobacco smoke, may alter collagen to produce neoantigens not present normally. Emphysematous lung may have a higher altered antigen density, and it might be possible to detect antigenic differences by immunofluorescent techniques between the normal and altered collagens and antisera from emphysematous animals or patients. The addition of antisera to cell lines, cultured with aldehydes

in order to produce altered collagen, may prove of great value in establishing which immunologic factors contribute to destruction of such cells, and if so, which aldehydes are intimately involved.

In vivo investigation of antibody will also be studied; such localization would provide important evidence that the antibodies to native or to altered collagen, though previously a result of the underlying process, bind to pulmonary cells in vivo, thus producing a viscious cycle by causing further damage. Antisera to complement components C_4, C_3, and C_2 (20) will also be used in these studies, since simultaneous binding of anti-collagen immunoglobulins (Ig) and of complement would strongly suggest a cytolytic action in vivo.

References

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10. Space and facilities available (when elsewhere than item 2 indicates, state location): Our biochemical laboratory has a total area of 1000 sq. ft. which includes 60 sq. ft. of office space. In addition, we have a tissue culture unit on an area of 150 sq. ft. Animals are maintained in central quarters, serving the whole institution. Major items of equipment include:1) Centrifuges; low speed (International), medium speed (Servall) and an ultracentrifuge (Beckman, Model L). 2) Fraction collectors (Buchler refrigerated, LKB), columns, recorders (Leeds & Northrup). 3) Spectrophotometer, Beckman Model DB, used for continuous flow monitoring of column effluents. 4) LKB column effluent monitor, used for proteins other than collagen. 5) Liquid scintillation counter, Packard. 6) Disc gel electrophoresis apparatus, analytical (Canalco). 7) Auto-analyzer (Technicon), used for automatic analysis of hydroxyproline. 8) Other standard equipment of a biochemical laboratory (balances, desk-top centrifuges, autoclave, etc.). 9) Fully equipped tissue culture unit (incubator, UV hoods, microscope, microphotography equipment).

11. Additional facilities required:

NONE

12. Biographical sketches of investigator(s) and other professional personnel (append):

See attached Pages 20 and 21

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See attached

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)

	% time	Amount
C. Michaeli, Ph.D., Assist. Prof. Res.	5%	**
C. Nevo, Ph.D. Assist. Res. Biochemist 111	100%	18,515.00
J. Belton, Ph.D., Assoc. Res. Immunol. I	35%	7,537.00
Technical		
D. Aiken, Staff Res. Assoc. III Step 5	100%	16,712.00
H. Scheuenstuhl, Staff Res. Assoc. III Step 5	100%	16,712.00
S. Inguito, Laboratory Assist. I	50%	4,858.00
N. Parkin, Secretary II Step 5	25%	2,677.00

**Salaries include 15% fringe benefits

Sub-Total for A 67,011.00

B. Consumable supplies (by major categories)

Radioactive materials, solvents for assays etc.	2,000.00
Tissue culture media, dishes, gas mixture	4,800.00
Glassware and chemicals	2,000.00
Animals, purchase and maintenance	3,000.00

Sub-Total for B 11,800.00

C. Other expenses (itemize)

Travel (Aspen Emphysema Conf.)	800.00
Publication costs	1,000.00

Sub-Total for C 1,800.00

Running Total of A + B + C 80,611.00

D. Permanent equipment (itemize)

Spectrofluorimeter (for DNA Determinations) Turner Model 430 3,900.00

Sub-Total for D 3,900.00

E. Indirect costs (15% of A+B+C) E 12,092.00

Total request 96,603.00

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	70,362	11,800	1,800	-0-	12,594	96,556.00
Year 3	73,881	11,800	1,800	-0-	13,122	100,603.00

1003540133

Justification for Budget Items

Personnel

Z. Nevo, Ph.D.--He is a highly trained biochemist and is well versed with tissue culture techniques which he learned in Dr. Dorfman's laboratory at the University of Chicago. He will conduct the biochemical assays on the cell cultures as detailed in this proposal.

J. Belton, Ph.D.--Dr. Belton is an electron microscopist who, in addition to his skill with transmission electron microscopy, is familiar with the powerful techniques of freeze-fracture and freeze-etching electron microscopy.

Technical:

D. Aiken--Has participated in the development of a radioimmunoassay technique for determination of collagen and antibodies to collagen. She will be involved in application of this method as described in the proposal.

H. Scheuenstuhl--Has been maintaining an excellent facility for tissue culture and will be indispensible for the continuation of the tissue culture studies proposed herein.

S. Inguito--Will be involved primarily with dish washing and general maintenance activities. Tissue culture operations generate a large amount of glassware that has to be cleaned and sterilized, and her function in the smooth functioning of our studies is extremely important.

N. Parkin--Will perform secretarial duties that have increased since we initiated the studies on effects of tobacco smoke on lung tissue and we anticipate a further increase in the load once the proposed projects proceed at full pace.

Technician for Immunofluorescence	50%	5,226

As stated on page 8 of this application, the immunofluorescent studies are proposed on a trial basis. To carry them out, a technician for immunofluorescence will be needed at a 50% effort.

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Career Development Award	5 K04 AM50205	21,735	1/1/71-12/31/75
Immunochemistry of Lung Collagen	HL-14759	250,000	3/1/72-2/28/76
Trauma Center Project	GM18470	40,000	8/1/71-7/31/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Dov Michaeli, Ph.D.

Signature Dov Michaeli Date 1/15/74

Telephone 415 666-1865
Area Code Number Extension

Checks payable to
The Regents of the University of California

Mailing address for checks
Gifts & Endowments
1487 4th Avenue
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Responsible officer of institution

Typed Name Sue Clark

Title Program Coordinator, Gifts & Endowments

Signature Sue Clark Date 1/28/74

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